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Interleukin-33 promotes the inflammatory reaction in chronic rhinosinusitis with nasal polyps by NF-κB signaling pathway

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Abstract. – OBJECTIVE: Interleukin (IL)-33 promotes T helper (Th2) immune response and may be involved in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). Using murine and human specimens, we evaluated the role of IL-33 in CRSwNP.

MATERIALS AND METHODS: To establish CRSwNP, Balb/c mice were sensitized with house dust mite, followed up by intranasal exposure to Staphylococcus aureus to stimulate the inflammatory response of nasal mucosa. The hematoxylin-eosin staining and total serum IgE were used to the successful construction of CRSwNP model. For mechanistic studies, we blocked mice with IL-33 and the Th2 cells counts in tissue were detected. Th2 cytokine expression of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment, were analyzed by qRT-PCR. Meanwhile, the relative mRNA and protein expression levels of NF-κB, MyD88 and TLR7 were detected after IL-33 blockade. To document the inflammatory response in patients with CRSwNP, The relative mRNA expression of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 in control individuals and patients with CRSwNP (chronic rhinosinusitis with nasal polyps) were analyzed by qRT-PCR.

RESULTS: The CRSwNP model was successfully constructed. After IL-33 blocked, the relative expression of IL-33 and Th2 cells counts were reduced significantly. CRSwNP mice showed overproduction of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 and IL-33 blockade inhibited the expression of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24. Furthermore, IL-33 blockade decreased the mRNA levels of NF-κB, MyD88 and TLR7, and also restrained the protein expression of them. On the other hand, patients' specimens with CRSwNP showed high levels of Th2 cytokines including IL-33, IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24.

CONCLUSIONS: CRSwNP is associated with overexpression of IL-33, with subsequent activation of Th2 immune response by NF-κB signaling pathway.

Key Words:
Interleukin-33, Rhinitis, Sinusitis, Nasal Polyps, Th2 Cells, NF-κB signaling pathway.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is an inflammatory disease of unknown etiology characterized by eosinophilic inflammation and overabundance of T-helper (Th) 2 inflammatory cytokines[1-3]. The disease cannot currently be cured by surgical or antibiotic treatments, which is burdensome and frustrating to patients[4-6]. The pathogenesis of this disease is unclear. It has been suggested that microbial triggers may overstimulate the inflammatory response of Th2 cells in CRSwNP, leading to chronic disease manifestations[7,8]. On the other hand, no direct association was found between the occurrence and development of nasal polyps, and specific pathogens or exogenous stimuli. It can, therefore, be assumed that host immune response is also a contributor to long-term inflammation in CRSwNP.

Overexpression of Th2 cytokines, such as Interleukin (IL)-4, IL-5, IL-13 and IL-22, is believed to play an important role in eosinophilic mucosal inflammation in CRSwNP[9,10]. This involves cell stimulation via the Interleukin 1 Receptor-like 1 Protein, which belongs to the IL-1 receptor family, but binds IL-33[11].

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IL-33 is a protein with the molecular weight of approximately 18 kD (270 amino acids). IL-33 is synthesized as a 30 kD precursor protein and is processed by caspase-1 to yield the mature protein of 18 kD[12]. Various tissues (digestive tract, respiratory tract, skin, lymphoid tissues, spleen, pancreas, kidney) are positive for IL-33 mRNA expression[13]. The IL-33 receptor Interleukin 1 Receptor-like 1 Protein is expressed at different levels in different blood immune cells[14].

Exogenous and endogenous stimuli (mechanical injury, infection, smoking, products of epithelial cell damage and necrosis) cause epithelial cells to release IL-33, which leads to subsequent inflammation[15]. Interestingly, biological effects of IL-33 depend on its length. As mentioned above, IL-33 is cleaved by caspase-1, which is activated in apoptotic cells. Cleavage by caspase-1 reduces the biological activity of IL-33. In contrast, the full-length IL-33 is inflammagenic[16,17]. Therefore, cell apoptosis inactivates IL-33 and prevents the development of the Type 2 immune response.

Animal models are very useful for studies of gene and protein function[18,19]. In this study, we utilized a murine model to test the involvement of IL-33 in the occurrence and development of CRSwNP. These observations were further supported by studies of patients' specimens.

Materials and Methods

Animals, Animal Groups, and Downstream Assays

Animal experiments were approved by the Animal Ethics and Experimental Committee of The First Affiliated Hospital of Harbin Medical University. Mice were purchased from the Center of Experimental Animals, The Second Affiliated Hospital of Harbin Medical University. Hundred and twenty specific pathogen-free Balb/c mice (female, 6 weeks old) were randomly assigned to each of the following experimental groups (10 animals per group): control group, CRSwNP group, and IL-33 blockade group. IL-33 blockade group's mice used IL-33 neutralizing antibodies purchased from R&D Company (Minneapolis, MN, USA). Mice were fed autoclaved and pelleted diet, and sterile water under the following conditions: 23°C, humidity of 50%, 12 h light and dark cycles, and quiet surroundings with minimal disturbance. Mice in all groups were tested at 12 weeks of age.

The model of nasal polyps was established as follows. The model of nasal polyps was built with a standardized house dust mite extract sensitization. The mice were subcutaneously injected by 2000 U/ml aluminum hydroxide containing 2% house dust mite extract 100 UL in first days. At 3rd, 5th, 7th days mice were intraperitoneally injected by 2000 U/ml aluminum hydroxide containing 2% house dust mite extract 100 UL for 3 times. At 9th, 11th, 13th days, they were injected by 4000 U/ml house dust mite extract 50 UL for 3 times; at first and 8th days, intraperitoneal injection of aluminum hydroxide containing 2% house dust mite extract 100 UL (2000 U/ml) for 2 times. At 15th, 16th, 17th days, mice were injected with 0.3% pentobarbital anesthetized mice after intraperitoneal injection of 0.3 ml, 4000 U/ml house dust mite extract 50 ul/slow nasal drops, 1 time/d. The control group was the same as the treatment group, but the drip and injection were the same amount of 0.1 mol/L phosphate buffered saline (PBS). Mice were sensitized with house dust mite, which induced allergic rhinitis. *Staphylococcus aureus* was used for inflammatory stimulation of nasal mucosa of experimental mice.

Patients

The use of patients' specimens was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University, Harbin, China. Our study included 45 patients and control individuals enrolled in Harbin, China, between April 2016 and August 2016. Thirty-five patients were diagnosed with CRSwNP, and 10 individuals with maxillary sinus cysts served as controls. Clinical specimens of patients' nasal mucosa were used for downstream analysis. Neither patients nor control individuals had a history of allergic diseases or positive skin prick test. None of study individuals received antibiotics or corticosteroids for 4 weeks prior to the enrollment.

Reagents

Mouse polyclonal antibodies to IgE and β-actin, and horseradish peroxidase-conjugated streptavidin (HRP-conjugated streptavidin) were purchased from Zhongshan (Beijing, China). Protease inhibitor cocktail, phenylmethylsulfonyl fluoride and chemiluminescence kit were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The RNeasy Mini kit and the First Strand cDNA Synthesis kit were purchased from Sinopharm Chemical Reagent (Shanghai, China).

Histological Staining

Surgically procured tissue specimens obtained from the CRSwNP group were formalin-fixed and paraffin-embedded. Paraffin sections that were 4-mm thick were stained with HE, Masson's trichrome according to the manufacturer's instructions. All parameters were assessed using an image analyzer (SP 500; Olympus, Tokyo, Japan).

Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples were drawn from the antecubital vein and collected into tubes containing heparin. After samples were centrifuged at 3000 rpm for 10 min at 4°C, the plasma was separated and stored at -80°C until the assay was performed. Plasma levels of IgE were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). As described previously[12], the procedures were performed according to the instructions from the manufacturer.

mRNA Isolation, cDNA Synthesis and qPCR

Total RNA (murine and human specimens) was extracted using the TRIzol reagent (Thermo Fisher, Beijing, China) according to manufacturer's protocols. The PrimeScript RT Master Mix (TaKaRa, Dalian, Liaoning, China) was used to reverse transcribe RNA into cDNA. Then, qPCR were carried out using the ABI 7900 system (Thermo Fisher, Beijing, China)) and SYBR Green PCR Master Mix (TaKaRa, Dalian, Liaoning, China). The expression of Th2 mRNA cytokines (IL-33, IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24) in nasal polyp tissue was compared with that in normal mucosa, following normalization to β-actin mRNA expression (reference gene). For quantification, we used the $2^{-\Delta\Delta}$Ct method.

In addition, we quantified mRNA expression of two subunits of the NF-κB, MyD88 and TLR7 in nasal mucosa tissue by semi quantitative PCR. For this purpose, we obtained 50 mg of tissue, which was flash frozen in liquid nitrogen, and pulverized into powder in 1 mL of TRIzol. cDNA was obtained using 1 μg of total RNA and RevertAid RT kit (Thermo Fisher). This cDNA served as the template for PCR. The following primers were used: NF-κB, forward primer 5'-ACCTGCAGTTCGATGCTGAT-3' and reverse primer 5'-CCTGTCACCAGGCGAGTTAT-3'; MyD88, forward primer 5'-CGACGCCTTCATCTGCTACT-3' and reverse primer 5'-ATGAGCTCGCTGGCGATGGA-3', TLR7, forward primer 5'-TCTCCAGACTCCTTCCATAG-3' and reverse primer 5'-GGAAGATCCTGTGGTATCTC-3', IL-4, forward primer 5'-CTGACGGCACAGAGCTATTGA-3', and reverse primer 5'-TATGCGAAGCACCTTGGAAGC-3'. IL-5, forward primer 5'-GAGCACAGTGGTGAAAGAGACCTT-3', and reverse primer 5'-ATGACAGGTTTTGGAATAGCATTT-3'. IL-13, forward primer 5'-GCCAGCCCACAGTTCTACAGC-3', and reverse primer 5'-GAGATGTTGCTCAGCTCCTCA-3'. IL-33, forward primer 5'-ACAACACAGACGTTCGTCTCATTG-3', and reverse primer 5'-GAACAGCACTTCTTCAAGGGTGA-3', IL-33, forward primer 5'-CCCAAGCTTATGAGACCTAGAATGAAGTAT-3', and reverse primer 5'-GCTCTAGATTAGATTTTCGAGAGCTTAAAC-3'. CCL-11, forward primer 5'-CATGAAGGTCTCCGCAGCACTTCT-3', and reverse primer 5'-CCAGATACTTCATGGAATCCTGC-3'. CCL-24, forward primer 5'-TCAGAAGTTTCTCTGTGTGGATTAC-3', and reverse primer 5'-GCGTTCTTAGATGGAATTCTCAG-3'. beta-actin, forward primer 5'-CGAAGCACAGTCAAAGAGAGGTA-3', and reverse primer 5'-GCTTCAGTCAAGAGAACAGGATG-3', PCR reaction was conducted as follows: denaturation step at 94°C for 60 s, annealing at 60°C for 45 s, primer extension are 72°C for 60 s. PCR was run for 40 cycles for mRNAs of interest, and for 35 cycles for the reference gene. PCR products were electrophoresed on a 1.5% agarose gel and visualized using ethidium bromide.

Protein Extraction and Western Blot

Specimens were lysed in the lysis buffer (0.1% SDS, 0.5% Triton X-100, 2% deoxycholic acid, 5 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl; pH 7.2). Lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies at 4°C overnight. The source of antibodies was rabbit IgG. Produced in rabbits immunized with purified, recombinant human IL-33, IL-4, IL-5, IL-13, IL-22, CCL-11, CCL-24, NF-κB, MyD88 and TLR7. Total IgG was purified by Protein A affinity chromatography. The following primary antibodies were: anti-IL-33, anti-IL-4, anti-IL-5, anti-IL-13, anti-IL-22, anti-CCL-11, anti-CCL-24, anti-NF-κB, anti-MyD88, anti-TLR7, anti-β-actin (all at a 1:1.000 dilution). This was followed by incubation with the secondary antibody (1 h at room temperature) and chemoluminescent detection.

Statistical Analysis

The GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis and graphic data representation. The one-way ANOVA as used for multiple comparisons, whereas independent t or x^2 tests were used to test the differences between two groups (respectively, for nominal and categorical data). The data are presented as mean ± SEM.

Results

CRSwNP Model Establishment in Balb/c Mice

The tested mice showed signs of inflammation. This was obvious on hematoxylin eosin (HE) staining, which showed nasal polyp tissue. Randomly selected 5 high magnification fields (× 400) demonstrated influx of inflammatory cells, including eosinophils (Figure 1A-B). Meanwhile, the sIgE expression levels in control group, CRSwNP group and IL-33 blockade group, were analyzed by ELISA. The expression level of serum total IgE in control mice was significantly lower than that in CRSwNP mice and IL-33 blockade inhibited the serum total IgE expression level compared with CRSwNP group (Figure 1C). The result showed CRSwNP model was successfully constructed.

The Expression Levels of IL-13 After CRSwNP Model Establishment and Th2 Cells in Tissue

To detect the effects of IL-33 blockade, the mRNA and protein expression levels of IL-13 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were analyzed and Th2 cells counts were detected in all groups. After the CRSwNP model was constructed at 12 weeks, the IL-33 expression levels were significantly increased. Expression of IL-33 mRNA and protein in control group and IL-33 blockade group were significantly lower than that in CRSwNP group at 12 weeks after CRSwNP model establishment (Figure 2A-C). Meanwhile, IL-33 blockade suppressed Th2 cells production (Figure 2D).

Cytokine mRNA Expression were Inhibited by IL-33 Blockade

Next, we quantified mRNA levels of Th2 cytokines. The mRNA expression levels of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were analyzed by qRT-PCR. CRSwNP mice showed upregulated levels of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 after the CRSwNP model constructed at 12 weeks and IL-33 blockade reverted this trend (Figure 3A-F).

The effects of IL-33 Blockade on the mRNA and Protein Expression of NF-κB, MyD88 and TLR7

We wanted to test whether the expression of the pro-inflammatory transcription factor inflammation-related factors was modulated in CRSwNP mice. The RT-PCR assay was used to analyze the mRNA levels of NF-κB, MyD88 and TLR7 in all groups. Our results demonstrated that the mRNA levels of NF-κB, MyD88 and TLR7 in CRSwNP group were significantly higher than those in control group (Figure 4A). However, after IL-33 blockade, the mRNA levels of NF-κB, MyD88 and TLR7 in IL-33 blockade

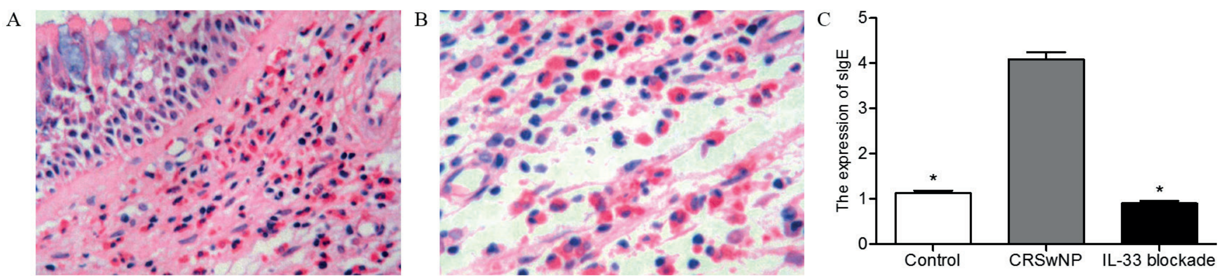

Figure 1. Histopathological sections of CRSwNP model establishment in Balb/c mice and sIgE expression levels. ***(A)*** Histopathological sections of tissues from CRSwNP model (× 100). ***(B)*** Histopathological sections of tissues from CRSwNP model (× 400). ***(C)*** The sIgE expression levels in control group, CRSwNP group and IL-33 blockade group were analyzed by Elisa. Data represent means ± SD, *p < 0.05 compared with CRSwNP group.

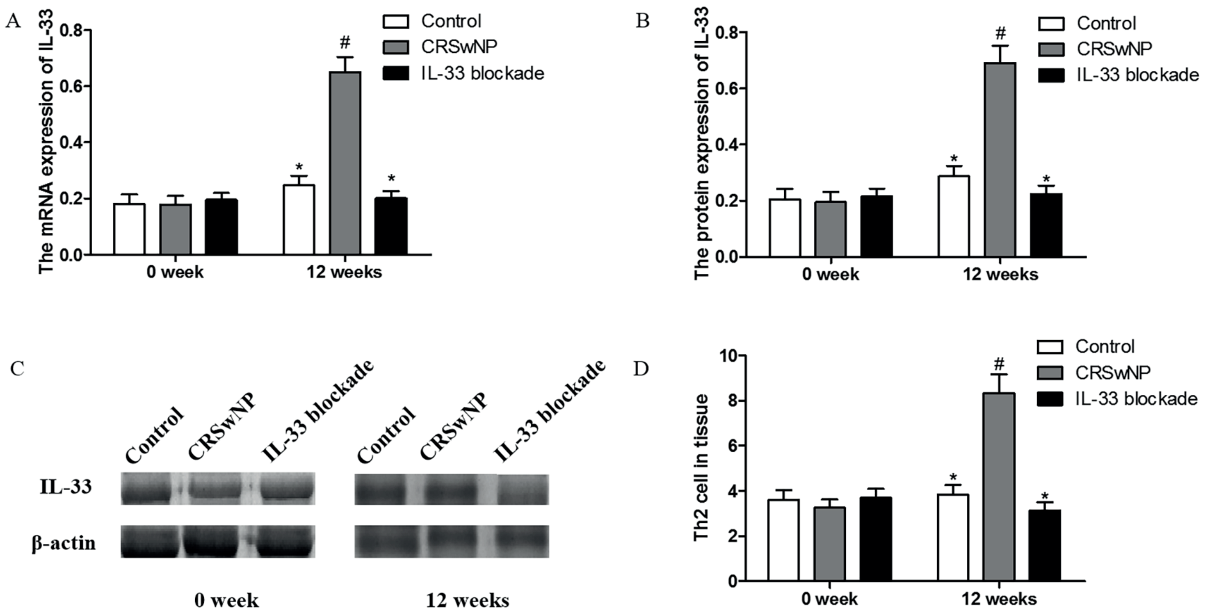

Figure 2. The expression levels of IL-13 after CRSwNP model establishment and Th2 cells in tissue. *(A)* The mRNA expression levels of IL-13 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were analyzed by qRT-PCR. *(B)* The mRNA expression levels of IL-13 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were analyzed by Western blot. *(C)* Relative quantification of Western blot analysis is depicted in the bar graphs. *(D)* The Th2 cells counts in control group, CRSwNP group and IL-33 blockade group. Data represent means ± SD, **p* < 0.05 compared with CRSwNP group; #*p* < 0.05 compared with CRSwNP group at 0 weeks.

group were lower than that in CRSwNP group (Figure 4A). To further confirm the mechanisms about the protein expression of NF-κB, MyD88 and TLR7, the Western blot analysis was conducted. Similar to the results of RT-PCR detection, we found that the CRSwNP mice remarkably increased the protein expression of NF-κB, MyD88 and TLR7 (Figure 4B and C). However,

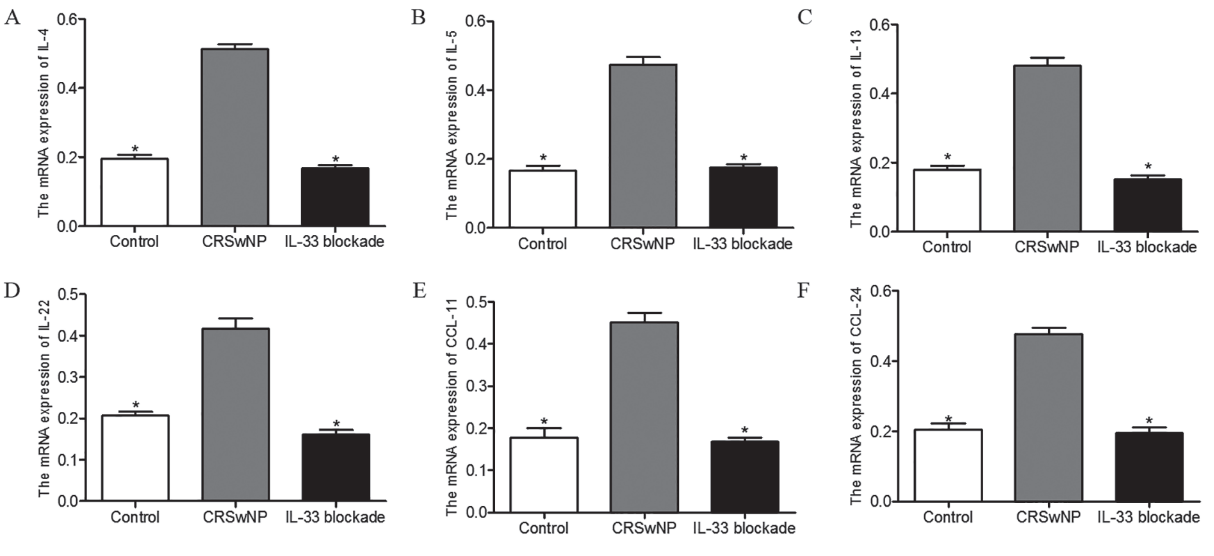

Figure 3. Cytokine mRNA expression in all groups. The mRNA expression levels of IL-4 *(A)*, IL-5 *(B)*, IL-13 *(C)*, IL-22 *(D)*, CCL-11 *(E)*, and CCL-24 *(F)* in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were analyzed by qRT-PCR. Data are mean ± SEM of 10 experimental animals per group. Data represent means ± SD, **p* < 0.05 compared with CRSwNP group.

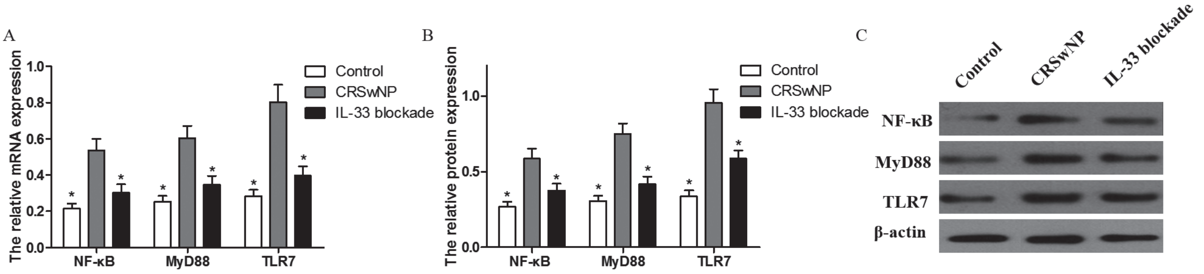

Figure 4. The effects of IL-33 blockade on the mRNA and protein expression of NF-κB, MyD88 and TLR7. ***(A)*** The relative mRNA expression levels of NF-κB, MyD88 and TLR7 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were analyzed by qRT-PCR. ***(B)*** The relative protein expression of NF-κB, MyD88 and TLR7 in control group, CRSwNP group and IL-33 blockade group at 12 weeks after CRSwNP model establishment were detected by Western blot. ***(C)*** Relative quantification of Western blot analysis is depicted in the bar graphs. Data represent means ± SD, $*p < 0.05$ compared with CRSwNP group.

after L-33 blockade, the protein expression of NF-κB, MyD88 and TLR7 in L-33 blockade group was clearly lower than that in CRSwNP group (Figure 4B and C).

The Relative mRNA Expression of Cytokine in Patients with CRSwNP and Control Individuals

Finally, to further confirm clinical relevance of our observations procured from murine studies, we analyzed specimens from 35 patients with CRSwNP and 10 control individuals. Specimens of patients with CRSwNP demonstrated overexpression of Th2 cytokines (Figure 5). This was true for IL-33, IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 (Figure 5).

Discussion

CRSwNP has been associated with Th2-driven immunity and the deficit of Th1 signals. The Th2 cytokines IL-4, IL-5, and IL-13 predispose to eosinophil influx[20]. Not surprisingly, the factors that induce the Th2 inflammatory pathway are being intensively studied[21]. One of such factors is IL-33, which is produced by epithelial cells and induces production of Th2 cytokines[22]. IL-33 is the cytokine of the IL-1 cytokine family. In the present study, we investigated the role of IL-33 in CRSwNP, using both a mouse model of this disease and patients' specimens. Our observations indicate that CRSwNP specimens, both murine and from patients, exhibit high expression levels of IL-25 and IL-33 mRNA. Further confirming IL-33 blockade suppressed Th2 cells production, we observed that CRSwNP mice showed upregulated levels of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 after the CRSwNP model constructed at 12 weeks and IL-33 blockade, reverted this trend.

At present, IL-33 is believed to activate pro-inflammatory transcription factor NF-κB and pro-inflammatory mitogen-activated protein kinases, thereby up-regulating production of cytokines and chemokines (IL-6, IL-8, granulocyte colony-stimulating factor), and promoting influx of inflammatory cells, such as neutrophils[12]. Our data showed the mRNA and protein levels of NF-κB, MyD88 and TLR7 in the CRSwNP group were significantly higher than those in the control group. However, after IL-33 blockade, the mRNA

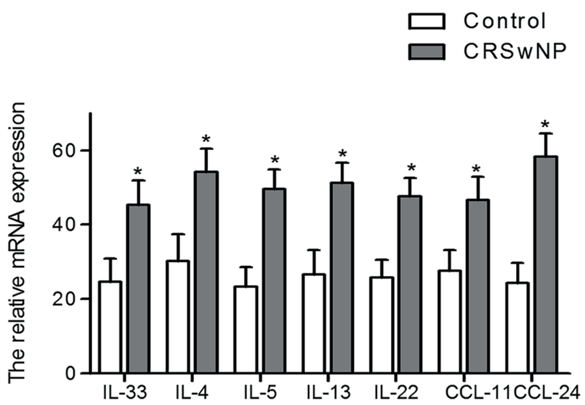

Figure 5. The relative mRNA expression of Cytokine in patients with CRSwNP and control individuals. The relative mRNA expression of IL-4, IL-5, IL-13, IL-22, CCL-11, and CCL-24 in control individuals and patients with CRSwNP (chronic rhinosinusitis with nasal polyps) were analyzed by qRT-PCR. Data represent means ± SD, $*p < 0.05$ compared with control group.

and protein levels of NF-κB, MyD88 and TLR7 in IL-33 blockade group were lower than that in CRSwNP group. It indicates that IL-33 may also promote the cytokine production by NF-κB signaling pathway.

It can be speculated that in susceptible individuals, contact of mucosal epithelia with environmental factors results in mucosal trauma and infection, and subsequent epithelial cell damage. The resulting release of IL-33 stimulates immune system. Studies, including this report, demonstrate that in nasal polyps, expression of Th2 cytokines is increased. Subsequently to the production of IL-4, IL-5, and IL-13, mucosal damage is aggravated.

Conclusions

We showed that CRSwNP was associated with overexpression of IL-33, with subsequent activation of Th2 immune response by NF-κB signaling pathway. Thus, IL-33 may be considered a potential drug target in CRSwNP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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